STUDIES ON CONDITIONS AFFECTING THE SURVIVAL IN VITRO OF A MALARIAL PARASITE (PLASMODIUM LOPHURAE)

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In 1912 Bass and Johns (2) reported a method for the cultivation of two species of malaria parasites of man. They stated that they observed in culture not only growth of ring forms to schizonts, but also the invasion of new red cells by merozoites. Unfortunately, they gave no figures or counts, and their only criterion for survival was the appearance of the parasites in stained films. In most of the subsequent work (27, 28, 33, 26, 14, 21, 19, 12) no development was ever observed beyond the growth of the original young forms into schizonts. Horovitz and Sautet (12) summarize their own and previous work by stating that development in the "cultures" is at best very mediocre, that it is as good in simple preparations of blood cells in dextrose-serum as in more complicated media, and that counts show that no multiplication of the parasite occurs. Bouilliez (3) tried the method of Bass and Johns with *Plasmodium inui* of the monkey. At 40°C. the parasites rapidly disintegrated, but when the preparations were held at about 22°C. segmenters could still be found after 6 days. Reports of similar experiments with avian malaria parasites are few. Sergent, Sergent, and Catanei (20) observed that canary blood infected with *P. relictum*, when mixed with equal parts of glycerin and 15 per cent citrate solution and held at 15–23°C., retained its infectivity for 3 days. Manwell and Hewitt (15) found that *P. praecox* could survive a few days in sealed tubes of heparinized blood at 25°C., but not at higher temperatures. Hewitt (11) obtained the slow and irregular development of young forms of *P. cathemerium* into schizonts when infected blood was spread over inspissated egg slants and incubated at 37°C. The merozoites which were formed were not liberated from their host cells and no infection of new red cells occurred. Gavrilov, Bobkoff, and Laurencin (9) observed that tissue cultures from the bone marrow of chickens infected with *P. gallinaceum* remained infective for 10 days at 37–38°C., but there was only very short survival when infected blood was added to tissue cultures of spleen or bone marrow from uninfected chickens. Hegner and Wolfson (10) prepared roller tube tissue cultures from the brain, lungs, and spleen of canaries heavily infected with *P. cathemerium* and obtained infections from these tissues after 8, but not after 15, days in culture at 37.5°C.

It is obvious from all these results that the cultivation of malarial parasites *in vitro* has hardly been approached. There seems to be no agreement concern-

ing even the simplest conditions favorable to the survival of the parasites *in vitro*. Bass and Johns (2) indicate that human malaria parasites must be anaerobic and that dextrose favors their survival. Manwell and Hewitt (15) stored *P. praecox* in sealed tubes, but Hewitt (11) states that *P. cathemerium* parasites seem to do better if spread in a very thin layer and hence under far from anaerobic conditions. Manwell and Hewitt (15) could not find any favorable effect of various concentrations of dextrose. Therefore it appeared desirable to undertake a somewhat systematic study of the effect of various *in vitro* conditions on the survival of malaria parasites.

Some care must be exercised in the interpretation of survival. For example, it is well known that many organisms can survive unfavorable conditions for a longer time at lower temperatures than at higher ones. This is true of malaria parasites too. *Plasmodium praecox* in citrated blood at 4–6°C. retained its infectivity for 5 days (13); *P. gallinaceum* in defibrinated blood at 0–5°C. remained infective for 21 days (9); and *P. knowlesi* and *P. inui* could be maintained in the frozen state at −76°C. for 70 days or longer (6). The brief survival of *P. praecox* (15) and of *P. inui* (3) at 25°C. but not at higher temperatures has already been noted. But certainly low temperature is not the kind of condition for survival which is of importance in attempts at the ultimate development of a culture method, for the malaria parasites of mammals and birds develop, in nature, at temperatures of 37.5 to 42 or 43°C. Very low temperatures are effective for the preservation of the parasites in a state of suspended animation for relatively long periods. But in the studies presented in this paper no effort has been made to obtain long preservation. The aim has been rather to study the comparative effects of a variety of agents and conditions on survival at temperatures which would be likely to bring about either development or rapid death of the organisms.

It is also necessary to establish suitable criteria for the determination of survival. Malaria parasites are typically non-motile. Something can be said as to their condition by observing their microscopic appearance in fresh and stained films. But, as Manwell and Hewitt (15) have already emphasized, the most objective and reliable criterion of survival is the ability of the parasites to infect a susceptible host. If the infectivity test is to be used for any extensive series of experiments, it is essential that the susceptible host be an experimental animal readily available in large numbers. It was largely for this reason that *Plasmodium lophurae* (5, 24, 25), a parasite capable of infecting chickens, was selected as the material for the experiments reported in the present paper. All of the experiments to be described were concerned with those stages only of the parasite which develop in the vertebrate host.

Methods

The Maintenance of Strains of Plasmodium lophurae.—A strain of this parasite was obtained through the kindness of Dr. L. T. Coggeshall and has been maintained by

passage every 6 days in baby chicks. At each transfer, 0.05 ml. of heparinized blood from the heart of a heavily infected chick is inoculated intracerebrally under ether anesthesia to each of a number of 2 day old chicks weighing about 25 gm. In such young chicks the intracerebral method of inoculation is fully as reliable in producing heavy infections, which reach their peak on the 5th or 6th day, as is the intravenous method used by Terzian (24) for 40 to 50 gm. chicks. Moreover, as judged by the minimum number of parasites required to produce an apparent infection, 2 day old chicks inoculated intracerebrally are considerably more susceptible than approximately 1 week old chicks inoculated intravenously (see section on infectivity).

A strain of the parasite has also been maintained in ducklings 1 to 3 weeks old (32). At each transfer, 0.2 to 0.5 ml. of heparinized blood from the heart of a heavily infected duckling 6 days after its inoculation is injected intracardially into each of 2 or 3 fresh birds. The first duck inoculated with heavily infected blood from baby chicks showed only a light infection, but since the third duck passage a heavy infection is regularly obtained which usually reaches its peak on the 5th day. By subinoculating on the 6th day, it is possible to maintain a high parasite level which at the same time permits most of the hosts to survive.

Preparation of Parasite Material for the Survival Tests.—From the heart of a 1 week old chick, infected 5 days previously with *P. lophurae*, it is possible to obtain 1 to 1.8 ml. of blood representing at the most only 0.3 to 0.6 ml. of red cells. About 10 ml. of blood can regularly be obtained under sterile conditions from the heart of a chicken weighing about 300 gm. Hence it seemed desirable to use birds of this size as a source of parasites for the survival tests. One chicken could then yield enough material for a considerable series of preparations. However, the chicken shows a marked age immunity toward *P. lophurae* (5, 22, 25). This difficulty has been overcome by the use of chickens injected intraperitoneally into the ventral, right posterior portion of the peritoneal cavity with 5 ml. per 100 gm. body weight of a 15 per cent by volume dilution, containing 0.85 per cent sodium chloride, of Higgins Eternal black writing ink (30). This amount of ink was administered on the day before inoculation with *P. lophurae*, on the day of inoculation, and for 2 or 3 days thereafter. Each chicken was inoculated intracardially with 0.8 to 0.9 ml. of pooled heparinized blood from heavily infected baby chicks. 4 or 5 days after inoculation blood was taken aseptically from the heart of a chicken showing a sufficiently heavy infection into a syringe containing an appropriate volume of heparin-sodium chloride solution (0.05 ml. per ml. of blood to be taken). The heparinized blood was centrifuged for 5 minutes, the plasma was drawn off, and the cells were resuspended in the proper volume of a special balanced salt solution (see section below).

When parasites from ducks were desired, a 2 or 3 week old duck on the 4th or 5th day of its infection was bled in the same manner as the ink-treated chickens, and the blood was treated in an identical way.

Media Used and Preparation of the Survival Tests.—It appeared useful to have a suitable balanced salt solution for the washing and suspension of infected red cells, as a diluent for various cell and organ extracts, etc. Since malaria parasites live within the red cell and since the interior of the red cell has a very different composition from the serum which bathes it, an attempt was made to construct a balanced salt solution based on available knowledge of the composition of red blood cells, and on certain general considerations. The composition of this solution, to be designated hereafter

as solution K, is given in Table I, together with the composition of Tyrode's and Locke's solutions. Both of the latter roughly approach the composition of serum, and it will be noted that solution K has a much higher potassium content, a higher phosphate content, and a lower pH, in keeping with the properties of red cells as compared with serum (1, 16–18, 23). The potassium content of solution K is 330 mg. per cent, as compared with a measured potassium content for goose erythrocytes of 230 mg. per cent and for goose serum of 11.5 mg. per cent (18). In the preparation of solution K, as well as of all other solutions, reagent salts, Pfanstiehl dextrose, and double-distilled water were used. Such solutions were sterilized by filtration through a Berkefeld N filter. In some of the experiments it was convenient to have sterile

TABLE I

The Composition of Solution K, and of Tyrode's and Locke's Solutions

	Solution K	Concentration		
		Solution K	Tyrode's	Locke's
	gm. per liter	*mM per liter*	*mM per liter*	*mM per liter*
NaCl	3.039	52.0	136.8	154.0
KCl	4.100	55.0	2.6	5.6
$NaH_2PO_4 \cdot H_2O$	0.690	5.0	0.4	—
K_2HPO_4	2.613	15.0	—	—
$NaHCO_3$	0.168	2.0	5.9	2.4
$CaCl_2$	0.166	1.5	1.8	2.1
$MgSO_4 \cdot 7H_2O$	0.370	1.5	—	—
$MgCl_2 \cdot 6H_2O$	0.407	2.0	0.5	—
d-Glucose	2.377	12.0	5.0	12.6
Total molarity		140.0	150.5	170.4
pH		7.2	7.3–8.0	7.4

concentrated solutions of the various constituents of solution K and to mix in sterile tubes the appropriate amounts of these and of sterile distilled water.

Heparin was the chief anticoagulant employed. A solution of 13.6 mg. of Connaught highly purified heparin was prepared in 50 ml. of 0.85 per cent sodium chloride solution. This was placed in small tubes and autoclaved, and the tubes were then capped with parafilm and stored in a refrigerator. In several experiments, solution K was modified so that it contained no calcium and, per liter, 5.0 gm. of sodium citrate and only 2.046 gm. of sodium chloride. This "citrate solution K" could then be used as the anticoagulant.

Early in the course of the work it became apparent that chicken red cell extracts greatly favored the survival of *P. lophurae*. These extracts were usually prepared in the following manner. Blood was taken aseptically from the heart of 1 to 2 month old chickens and was defibrinated by shaking with glass beads (or, more rarely, an anticoagulant was used). The blood was then centrifuged 5 minutes at a low speed and the serum (or plasma) drawn off. The cells were frozen by immersion of the

centrifuge tube in an alcohol-dry ice mixture, and they were then allowed to thaw slowly in an incubator at 40°C. When they had thawed, the resulting dark red viscous liquid was mixed with an appropriate volume of diluent, as solution K, and the mixture was centrifuged at the high speed of an ordinary centrifuge for 1/2 to 1 hour. The clear red supernatant liquid was then drawn off and stored in a refrigerator at 8–9°C. until used. Warburg (31) showed that a single freezing and thawing of avian red cells did not diminish their respiration and that even the nucleus-free fluid showed a slight oxygen consumption. Red cell extracts were prepared 1 to 3 days before use. The term "dilute red cell extract" in the tables giving the experimental results, refers to extracts in which the volume of diluent was at least twice the volume of red cells. For concentrated extracts, a volume of diluent equal to that of the cells was used. With smaller volumes of diluent, practically no nucleus-free supernatant liquid could be obtained, since the frozen-thawed cells after dilution and centrifugation had an appreciably greater volume than the untreated cells. For this reason, attempts to get very concentrated chicken erythrocyte extracts free from the cell nuclei have thus far failed. However, in some of the work, the frozen-thawed red cells themselves, either undiluted or slightly diluted, were used.

Chick embryo extracts were prepared as follows. 10 day old embryos were removed aseptically, rinsed in a small dish of solution K, and their eyes removed. The embryos were then transferred to another sterile dish in which they were minced. The minced tissue was suspended in solution K, used in the proportion of 1.5 ml. per embryo, and the material was centrifuged 5 minutes at low speed. The opalescent supernatant liquid constituted the extract. Embryo extracts were prepared 1 day before use and were stored in a refrigerator.

The liver extract used for the experiments given in series 4, 5, 6, and 7 of Tables VIII and IX was made by extracting overnight 250 gm. of ground chicken livers (previously stored in the frozen state) in 50 ml. of citrate solution K at a pH of 6.4. The mixture was heated in a water bath to 65–70°C. and held at this temperature for 10 minutes. It was then squeezed through gauze and filtered through paper to give a clear dark amber filtrate. The pH was adjusted to 6.7, the material was sterilized by Berkefeld filtration and was stored in the refrigerator. After a few days storage, some sediment appeared. Other liver extracts were prepared in an essentially similar manner.

All extracts refer to chicken material, unless otherwise stated. Parasites from chicks were used only with chicken red cell extracts, parasites from ducks only with duck red cell extracts.

In the preparation of the survival tests, the media to be tried were pipetted into appropriate sterile containers. The parasite suspension was then prepared as rapidly as possible and the media were inoculated with it. All the tests, except a few preliminary ones, were incubated at temperatures of from 39.5–42°C. Small Erlenmeyer flasks (Fig. 1) proved to be the simplest and most effective type of container. These 25 ml. pyrex flasks, previously cleaned with cleaning fluid, 10 per cent nitric acid, water and distilled water, and dried, were plugged with gauze-lined cotton plugs. Over the mouth of each flask was placed a glass vial and the whole was sterilized by dry heat. Each such flask usually received 2 ml. of medium and 0.2 ml. of parasite suspension. The glass vial was then replaced and sealed to the side of the flask with

a ribbon of parafilm. This method of capping and sealing the flasks effectively prevented drying out of the contents and reduced the chances for contamination from the mouth of the flask. Most of the flasks were opened daily for examination, being held at room temperature only as long as absolutely necessary, and were then resealed with a fresh ribbon of parafilm. When a flow of gas through or over the medium was desired, a 7.5 × 2.5 cm. vial or a 50 ml. Erlenmeyer flask was used. This was equipped with a rubber stopper bearing gas entrance and exit tubes, each with a bulb stuffed with cotton. The gas was passed through distilled water and through a trap bottle before entering the experimental container.

In many of the experiments, one-third to one-half of the volume of medium was replaced daily with fresh corresponding medium which had been stored in the refrigerator. This was accomplished by gently tilting the flask and drawing off a portion of the supernatant liquid, being careful not to suck up the sediment of red cells and parasites. The quantity of liquid drawn off was measured and an equal quantity of fresh medium, first warmed to 38–40°C., was then added.

Determination of Survival.—

Morphology of the Parasites.—The study of dried films stained in the usual manner with Giemsa has proved to be more valuable than the examination of freshly prepared wet films. In most experiments, a Giemsa-stained dried film from each survival test was prepared and examined daily. When most of the parasites presented much the same appearance as in blood smears from an infected animal they were rated as good (G in the tables). When about half appeared degenerate, *i.e.* either the cytoplasm stained very darkly or the nuclei failed to stain, or both, the appearance was rated fair (F in the tables), and when most of the parasites appeared degenerate it was rated poor (P). It will be apparent from an examination of the results presented in the tables that the appearance of the parasites was not always a dependable indication as to their survival as judged by infectivity.

The Parasite Number.—In some experiments, parasite counts were made per 1000 red cells on the original parasite suspension and on the smears prepared from the survival tests. Since an effort was made to count only normal appearing parasites, this second criterion of survival involves also the first one. However, in favorable preparations, during the first 2 or 3 days of incubation many of the parasites do appear normal and some useful information can be obtained from the counts. Hemocytometer counts showed that in these preparations the number of red cells per c. mm. did not decrease significantly until the 3rd, and occasionally not until the 4th day. Hence the relative parasite number during the first 2 days is a dependable indication of the absolute parasite number.

Infectivity.—Infectivity is a relative thing and has to be defined in terms of the actual operations used. For example, Boyd (4) and Demidowa (7) showed that with small numbers of *P. praecox* the length of the prepatent period varied inversely with the number of parasites inoculated. Boyd concluded that no apparent infection was produced by less than 1000 parasites, but Demidowa, by injecting a much larger number of birds and following them for a much longer time, found that even a single infected red cell could produce a visible infection (2 birds out of 70 injected). Obviously no comparisons can be made between an experiment in which conditions are such as to detect an infection produced by one or a few parasites surviving out of

millions originally present, and an experiment in which no infection will be detected unless at least several hundred or several thousand infective parasites are present in the inoculum. Fulton (8) states that 10 ml. of blood infected with the monkey parasite *P. knowlesi* and mixed with 0.1 ml. of 50 per cent glucose solution, was still infective 12 days later when it reached the United States from England. Temperature conditions during transit are not stated, but in any case it is known that only a very few *P. knowlesi* injected into a *rhesus* monkey will produce a fatal infection. Since the original blood probably contained a very great number of parasites, the percentage survival may actually have been almost infinitesimal.

For these reasons it was necessary to discover the minimum numbers of *P. lophurae* which would give infections apparent under the arbitrary standard conditions used to determine the infectivity of survival tests. These conditions were as follows. For each test of infectivity two 2 day old chicks were inoculated intracerebrally under ether anesthesia with a measured sample of material. On the 7th day and again on the 11th day after injection a Giemsa-stained blood smear was prepared from each chick. Each smear was examined until parasites were found, but for a period not exceeding 5 minutes. If parasites were found in the 7 day smear the infectivity was designated ++; if they were not found in the 7 day smear but were found in the 11 day smear the infectivity was designated +; if they were not found in a 5 minute examination of both the 7 day and 11 day smears the infectivity was designated −.

The minimum numbers of parasites required to produce infection under these standard conditions were determined by making suitable dilutions in 0.85 per cent salt solution of freshly drawn heparinized or citrated blood on which a red cell and parasite count was made. Two chicks were inoculated intracerebrally with 0.03 ml. of each dilution and blood smears were made and examined 7 days and again 11 days later. Seven experiments were performed using blood from infected chicks, and 5 with blood from infected ducks. The results are given in Table II. The extent of variation was not great, especially when one considers that only two chicks were used to test each dilution, and that the chicks were not of a genetically uniform stock. It can be said that, on the average, about 2000 parasites in chicken erythrocytes were required to produce an infection apparent on the 7th day, and about 200 parasites to produce one just apparent on the 11th day. When the parasites were present in duck erythrocytes, much larger numbers were required to produce infection in baby chicks, as was to be expected. In 4 out of the 5 experiments, about 100,000 was the minimum number giving an infection apparent on the 7th day. At least about 10,000 parasites had to be inoculated to produce an infection just apparent on the 11th day. It is interesting to note that Terzian (24), using 50 gm. chicks inoculated intravenously, could not detect any infections if an inoculum of less than 50,000 parasites (from chicks) was used, even though smears were made for 18 days after inoculation. This of course explains his inability to demonstrate latent infections in recovered birds. By the use of the intracerebral technique with 2 day chicks it has been possible to demonstrate parasites in the blood of a rooster 6 months and 1 year after recovery from the initial infection.

Since most of the survival tests contained originally 50,000 to 100,000 parasites per c. mm., it is obvious that an infection apparent in 7 days after the inoculation of 50 c. mm. could have been produced by only 2000 parasites or 40 per c. mm., a survival

of less than 0.1 per cent. In the more recent experiments, suitable dilutions have been made to test for the survival of 40, 20, and 10 per cent of the organisms originally present. For example, in testing for 20 per cent survival of parasites in chick blood, the dilution was prepared in such a way that a dose of 20 c. mm. contained 10,000 parasites, on the basis of the original number present. If one or both of the chicks inoculated with this dose showed infection on the 7th day, it could be concluded that at least 20 per cent of the parasites were still infective. In a similar test using parasites in duck blood it would be necessary to have 500,000 parasites in the dose of 20 c. mm. In all the dilution tests, a dose of only 20 c. mm. was used, as this was never followed by leakage on withdrawal of the needle from the cranium.

TABLE II

The Minimum Numbers of Plasmodium lophurae Required to Give an Infection Apparent, in a 5 Minute Search of a Stained Blood Film, within 7 and within 11 Days after the Intracerebral Inoculation of 1 to 2 Day Old Chicks with 0.03 Ml. of Appropriate Dilutions in Saline of Freshly Drawn, Parasitized Blood

Source of infected blood	Experiment No.	Minimum No. of parasites giving infection in 7 days	Next lower No. tested	Minimum No. of parasites giving infection in 11 days	Next lower No. tested
Chicks	1	3,000	300	3,000	300
	2	2,000	1,000	200	—
	3	1,000	500	200	—
	4	600	300	250	—
	5	7,000	700	175	90
	6	850	213	213	—
	7	2,500	640	160	—
Ducks	1	150,000	15,000	15,000	1,500
	2	75,000	7,500	7,500	750
	3	170,000	87,000	11,000	—
	4	980,000	240,000	31,000	15,000
	5	150,000	74,000	37,000	18,000

In the earlier experiments it was found that infectivity generally remained high during the first 2 days of survival *in vitro*. For this reason, and in order to save on the number of chicks used, in the later experiments infectivity tests were not begun until the 3rd day.

Exflagellation of the Male Gametocytes.—In order to observe this, a drop of material from the survival test was placed between slide and coverslip and allowed to stand at room temperature for 20–25 minutes. It was then examined for 5 minutes with a 4 mm. objective and 15 × oculars, and the number of actively motile exflagellants seen was determined. When none was found in 5 minutes, the examination was usually prolonged to 10 minutes. It may be noted that 15–20 minutes is the time which usually elapses before exflagellation can be seen in a drop of blood removed from an infected host and held at room temperature. This criterion for survival is fully as objective as the criterion of infectivity and very much simpler to carry out.

Although it has thus far been used in only a few experiments it promises to yield information as reliable as that obtained from the infectivity tests. It is possible that conditions which are best for the survival and development of male gametocytes are not best for the asexual forms. This would be a most interesting fact to determine.

RESULTS

Effect of the Balanced Salt Solution Used for Diluting

Solution K was originally prepared with only 8 millimols of glucose per liter and a proportionately higher NaCl and KCl concentration (1 millimol of salt being considered as the osmotic equivalent of 2 millimols of glucose). In this form the solution was tested against Locke's and against Tyrode's solution by mixing a little infected blood with each solution in a small test tube and later observing the appearance and infectivity of the material. For example in one test, on the 2nd day of incubation at 36°C., the parasites in Locke's solution presented a poor appearance and a − infectivity, while those in solution K had a fair appearance and a + infectivity. Again, in another test, in Locke's solution on the 2nd day the appearance was poor and the infectivity +, while in solution K the appearance was fair and the infectivity ++. Similar results were obtained in comparing solution K with Tyrode's solution.

In another experiment, the same amount of the same parasite suspension was inoculated to each of 6 tubes containing the following materials: (1) Locke's solution; (2) Locke's solution with an anti-chick red cell rabbit serum; (3) the same as (2) plus complement (guinea pig serum); (4) solution K; (5) solution K with the anti-chick red cell rabbit serum; and (6) the same as (5) plus complement. After 1 day at 36°C. the infectivity was ++ in tubes 1 and 4; in tubes 2 and 5 there was partial hemolysis, and the infectivity of tube 2 was − while that of tube 5 was ++; in tubes 3 and 6 there was apparently complete hemolysis, and the infectivity of tube 3 was − while that of tube 6 was +.

Effect of the Degree of Aeration

Some typical results obtained on this matter are summarized in Table III. The exposure of *P. lophurae* to air has evidently a very favorable effect on their survival *in vitro*. This was always the case, whether the extent of aeration was increased by spreading the parasites in a thin layer, by passing a current of air, or by suspending them in a semisolid gel. In preparations 2 and 3 of Table III, the melted 2 per cent agar was brought to a temperature of 45°C. and added to the tubes after all the other ingredients had been put in. The contents of the tubes were stirred vigorously and the tubes were plunged into a beaker of ice water for a minute or two. This rapidly set the medium into a semisolid gel in which the parasites were uniformly distributed. It is also evident from Table III that a high oxygen tension, as in almost pure O_2, or 95 per cent O_2 and 5 per cent CO_2, was detrimental. In several other experi-

TABLE III

Effects of Aeration and High Oxygen Tension on the Survival of Plasmodium lophurae at 39.5–40°C.

No.	Type of preparation	Appearance* and infectivity** after days:				
		1	2	3	4	5
1*a*	Dilute frozen-thawed red cell extract in solution K. 1 ml. in small test tube + 0.2 ml. parasite suspension. Height of liquid 15 mm.		F++	P−		
1*b*	Same medium as for 1*a*. 2.5 ml. in 25 ml. Erlenmeyer flask + 0.5 ml. same parasite suspension. Height of liquid 4 mm.		G++	G++		
2	4 ml. dilute heat-hemolyzed red cell extract in solution K + 0.5 ml. parasite suspension first partly hemolyzed with hypotonic red cell extract and then resuspended in isotonic red cell extract + 0.5 ml. 2% agar in isotonic NaCl solution. Column in tube 4 cm. high.	G+† G+ F−	F+ F− F−			
3	5.6 ml. dilute heat-hemolyzed red cell extract + 1.6 ml. parasite suspension + 0.8 ml. 2% agar in isotonic NaCl solution. Column in tube 4 cm. high.		G++† F++ F+			
4*a*	5.2 ml. dilute frozen-thawed red cell extract in solution K + 0.8 ml. parasite suspension in vial to depth of 17 mm. Current of air bubbled through.	G++	G++	F++	F+	P+
4*b*	Same as 4*a* but no air bubbled through.	G++	F+	P−	P−	
5*a*	2.5 ml. frozen-thawed red cell suspension + 1 ml. embryo extract + 0.3 ml. liver extract + 1.2 ml. solution K. In vial with air bubbled through.	G	F+	F+		
5*b*	Same as 5*a* but no air bubbled through.	G	F+	P−		
6*a*	1.5 ml. embryo extract + 0.5 ml. liver extract + 1.5 ml. 4% agar to make solid gel in a 50 ml. Erlenmeyer flask. Added 1 ml. frozen-thawed red cells + 0.4 ml. parasite suspension. Stream of CO_2-free air passed over surface.		G	G++		
6*b*	Same as 6*a* but oxygen passed over.		G	F−		
7*a*	3.1 ml. dilute red cell extract in solution K + 0.4 ml. embryo extract + 0.1 ml. liver extract + 0.4 ml. parasite suspension in 50 ml. Erlenmeyer flask. Mixture of 95% O_2, 5% CO_2 passed over surface.	G	G++	F−	P−	
7*b*	Same as 7*a* but mixture of 95% air, 5% CO_2 passed over surface.	G	G++	G++	F++	

* In this and all subsequent tables,

G = all or most of parasites appear normal;

F = some of the parasites appear normal;

P = few or none appear normal.

** In this and all subsequent tables,

++ = infection apparent 7 days after inoculation of 2 day old chick intracerebrally with 0.05 ml. of material;

+ = infection apparent 11 days after inoculation, but not 7 days after;

− = no infection after 11 days.

† In Experiments 2 and 3, samples were taken from the top, middle, and bottom of the column of semisolid gel in the tube. Results are given in that order.

ments, no difference could be detected between survival when air was passed through and survival when a mixture of 50 per cent air and 50 per cent oxygen was passed through.

The Effects of Glucose, Glycogen, and Glutathione

Bass and Johns (2) found that glucose and maltose, but not a series of other sugars and related compounds, favored the survival of *P. falciparum*. Since then, no other detailed experimental work on the problem has been reported. Fulton (8), however, measured the effect of various sugars on the rate of respiration of suspensions of *P. knowlesi*. Only glucose, levulose, maltose, mannose, and glycerol produced an increase in oxygen uptake as compared with that of parasites suspended in sugar-free medium.

The results of several typical experiments on the effects of added glucose on the survival of *P. lophurae* are given in Table IV. In all the preparations red cell extract and embryo extract were present, so that carbohydrate substrates were available apart from the glucose added, or not, with the solution K used in making the red cell extract. It is apparent, nevertheless, that added glucose favored survival (3 *a*, *b*; 7 *a*, *b*), that the presence of 12 millimols of glucose per liter was more favorable than 8 millimols per liter (2 *a*, *b*), and that a still higher concentration of glucose, 24 millimols per liter, was harmful (1 *a*, *b*; 3 *b*, *c*). This latter effect may have been due to a toxic impurity in the glucose.

Glycogen, at concentrations by weight approximately equivalent to the favorable concentrations of glucose, could replace glucose and, in fact, was even better than glucose in most of the tests conducted (4 *a*, *b*, *c*; 5 *a*, *b*, of Table IV). It is interesting to note in this connection that glycogen did not stimulate the respiration of the suspensions of *P. knowlesi* (8).

The results with the addition of glucose and glycogen showed up best when fairly dilute red cell extracts were used. With such extracts, a consistently favorable effect of added glutathione could also be demonstrated (5 *a*, *c*; 6 *a*, *b*, of Table IV).

The Effects of Renewing the Medium and of the Density of Parasites and Red Cells

Repeated trials showed that, other conditions being the same, survival for several days longer could be obtained if one-third to one-half of the medium was drawn off daily and replaced by fresh medium which had been stored in the refrigerator. When this was done, the contents of the flasks would retain a rather bright red color, instead of getting decidedly brownish, as in flasks in which the red cell extract medium was not renewed. It would probably be even better to renew all of the medium daily, but with the flat shallow layers of liquid which had to be used, it was impossible to draw off more than half of the medium and still avoid sucking up some of the sedimented red cells.

TABLE IV

Effects of Glucose, Glycogen, and Glutathione on the Survival of Plasmodium lophurae

(All preparations held in 25 ml. Erlenmeyer flasks at temperatures of 39.5–40°C., except for 7 *a* and 7 *b*, which were kept at 41–41.5°C.)

No.	Type of preparation	Appearance and infectivity* after days:					
		1	2	3	4	5	6
1 *a*	1.9 ml. dilute red cell extract in solution K but with 8 mM glucose per liter, NaCl and KCl raised proportionately, + 0.1 ml. embryo extract + 0.15 ml. parasite suspension.		G++	F+	P−		
1 *b*	Same as 1 *a*, but 24 mM glucose per liter, NaCl and KCl lowered proportionately.		G−	P	P		
2 *a*	1.8 ml. dilute red cell extract in solution K but with 8 mM glucose per liter, NaCl and KCl raised proportionately, + 0.2 ml. embryo extract + 0.2 ml. parasite suspension.		G++	F++	P−		
2 *b*	Same as 2 *a* but standard solution K with 12 mM glucose per liter.		G++	F++	F++		
3 *a*	1.84 ml. dilute red cell extract in solution K with glucose omitted, NaCl and KCl raised proportionately + 0.16 ml. embryo extract + 0.2 ml. parasite suspension.	F	F++	P−			
3 *b*	Same as 3 *a* but standard solution K with 12 mM glucose per liter.	G	F++	F+	P−		
3 *c*	Same as 3 *a* but with 24 mM glucose per liter, NaCl and KCl lowered proportionately.	G	F−	P−			
4 *a*	1.8 ml. dilute red cell extract in solution K with glucose omitted, NaCl and KCl raised proportionately + 0.16 ml. embryo extract + 0.04 ml. liver extract + 0.2 ml. parasite suspension.		G++	F++	F−	P	
4 *b*	Same as 4 *a* but using solution K with glucose replaced by 0.34% glycogen, NaCl and KCl raised proportionately.		G++	G++	F++	F−	
4 *c*	Same as 4 *b*, but 0.18% glycogen.		G++	G++	F++	F−	
5 *a*†	1.8 ml. dilute red cell extract in solution K + 0.16 ml. embryo extract + 0.04 ml. liver extract + 0.2 ml. parasite suspension.		G	G++	F+	P−	

TABLE IV—*Concluded*

No.	Type of preparation	Appearance and infectivity* after days:					
		1	2	3	4	5	6
5 *b*†	Same as 5 *a*, but glucose replaced by 0.18% glycogen, NaCl and KCl raised proportionately.		G	G++	F++	P+	
5 *c*†	Same as 5 *a* + 0.2% glutathione.		G	G++	F++	P++	
6 *a*†	1.84 ml. dilute red cell extract in 1 part serum + 3 parts solution K with glucose replaced by 0.2% glycogen, NaCl and KCl raised proportionately + 0.16 ml. embryo extract + 0.2 ml. parasite suspension.		G	G++	F++	P+	
6 *b*†	Same as 6 *a* + 0.1% glutathione.		G	F++	F++	P++	
7 *a*†	1.64 ml. dilute red cell extract in solution K with glucose omitted, NaCl and KCl raised proportionately + 0.16 ml. embryo extract + 0.2 ml. parasite suspension.	G	G	F++	F+	P+	P+
7 *b*†	Same as 7 *a* but with standard solution K with 12 mM glucose per liter.	G	G	F++	F++	P++	P+

* See footnotes, Table III.

† In these flasks, about 0.8 cc. of the medium was replaced daily with medium stored in the refrigerator.

In several tests, the contents of a flask were transferred to a centrifuge tube and centrifuged 5 minutes. The liquid was then drawn off and the sediment suspended in fresh medium. This procedure, perhaps because of the centrifuging, resulted in the rapid death of the parasites.

It was also observed in various experiments that poor survival was obtained if the parasite suspension used had a very high absolute parasite number. An experiment designed to test this and the preceding point is illustrated in Table V. Of the two flasks which each contained 2×10^5 parasites per c. mm., the medium was partially renewed daily in one, which gave a ++ infectivity on the 5th day, but not in the other, which already on the 3rd day gave a − infectivity. Of the other four flasks, in two the parasites only were decreased, the density of red cells being kept about constant, and in two both the red cells and parasites were decreased. The medium was not renewed in any of these flasks. It is evident that the smallest density of parasites gave the longest survival in both cases, the effect of total red cell concentrations being relatively slight. However, the best infectivity of all, on the 5th day, was obtained in

the flask which had the highest parasite density but had received fresh medium daily. The relative numbers of normal looking parasites gave results somewhat parallel to those based on infectivity. Especially noteworthy are the figures for flasks 1, 4, and 6 on the 3rd day.

TABLE V

Effects of the Density of Parasites and Red Blood Cells and of Renewal of the Medium on the Survival of Plasmodium lophurae

(All preparations held in 25 ml. Erlenmeyer flasks at 39.5–40°C. Each flask contained 1.8 ml. of the following medium: One part of frozen-thawed red cells extracted with 3 parts solution K and nuclei removed by centrifugation. To 15 ml. of this extract added 1 ml. embryo extract. In flask No. 1 0.8 to 1.0 ml. of medium was replaced daily with medium stored in the refrigerator.)

Flask No.	Parasite suspension added	Uninfected red cell suspension added	Parasites per c. mm.	Red cells per c. mm.	Parasites per 1000 red cells after days:				Appearance and infectivity* after days:					
					0	1	2	3	1	2	3	4	5	6
	ml.	*ml.*												
1	0.2	0	2×10^5	3.2×10^5	674	522	336	92	G	G	F++	P++	P++	
2	0.1	0.1	1×10^5	3.5×10^5	286	170	208		G	G	F++	F+		
3	0.05	0.15	0.5×10^5	3.6×10^5	140	102	90		G	G	F++	F++	P+	−
4	0.2	0	2×10^5	3.2×10^5	674	468	330	36	G	G	F−	P−		
5	0.1	0	1.1×10^5	1.7×10^5	674	468	408		G	G	F++	F++	P−	
6	0.05	0	0.6×10^5	0.9×10^5	674	458	428	100	G	G	F++	F++	F+	−

* See first footnote, Table III.

Effects of Serum, Plasma, and Embryo Extract

In a series of experiments using relatively concentrated red cell extracts prepared in varying concentrations of serum and citrated plasma, observations were made on exflagellation as well as on infectivity and relative parasite numbers. The results of some of these experiments are summarized in Tables VI to IX. All the preparations were held in 25 ml. Erlenmeyer flasks at 41.5–42°C. Except for preparation 1 *e*, each flask received originally 2 ml. of medium and 0.2 ml. of parasite suspension. Every day 0.6 to 1.0 ml. of medium in each flask was replaced by corresponding medium which had been stored in the refrigerator. It will be noted that the original density of parasites was held at about 30,000 to 50,000 per c. mm. for suspensions from chicks, and at about 60,000 to 80,000 per c. mm. for suspensions from ducks. Table VI shows that survival was very short in citrated chicken plasma (1 *d*) but was prolonged if the plasma was diluted somewhat and contained red cell extract (1 *c*). Chicken serum alone gave a + infectivity even on the 6th day (1 *b*),

while the infectivity of the preparation (1 *a*) with diluted serum and red cell extract was gone after the 5th day. However, the latter preparation had a higher infectivity on the 4th day than did the serum alone and also gave

TABLE VI

Effects of Serum, Plasma, and Red Cell Extract on Survival of Plasmodium lophurae

No.	Medium	Parasites per c. mm.	Red cells per c. mm.	Infectivity* after days:				Exflagellants† after days:		Parasites per 1000 red cells after days:			
				3	4	5	6	5	6	0	1	2	3
1 *a*	Red cell extract—8 ml. frozen-thawed cells in 6 ml. citrate solution K + 2 ml. serum.	.5 × 10^5	2.3 × 10^5	20% ++	10%+ ++	+	−		0	226	206	146	70
1 *b*	Serum—same as used for 1 *a*.	.5 × 10^5	2.3 × 10^5	20% ++	10%+ +	+	+	1	0	226	157	78	36
1 *c*	Red cell extract—8 ml. frozen-thawed cells in 6 ml. citrate solution K + 3 ml. citrated plasma (1 part citrate solution K to 3 parts blood).	.5 × 10^5	2.3 × 10^5	20% ++	10% ++	−	−		0	226	230	168	74
1 *d*	Citrated plasma—same as used for 1 *c*.	.5 × 10^5	2.3 × 10^5	10% −	−	−	−			226	172	48	
1 *e*	Mixed 1 ml. of red cell-embryo extract (8 ml. frozen-thawed cells in 14 ml. solution K—to 10 ml. extract added 1 ml. embryo extract) with parasite suspension and 1 ml. of heparinized plasma. Allowed mixture to clot. Then added 0.8 ml. of the red cell-embryo extract. Clot ground up for test on 5th day.	.7 × 10^5	2.9 × 10^5			++		10		240		226	100

* 20%++ signifies an infection apparent 7 days after the inoculation of the chick with a dilution such that the chick received, on the basis of the number of parasites originally present, a total dose of 10,000 parasites if contained in chick red cells, or of 500,000 parasites if contained in duck red cells, and indicates that at least 20% of the parasites originally present were still alive. (See section on infectivity under methods.) 20%+ signifies, under similar conditions, an infection apparent after 11 but not after 7 days, and 20%− no infection after 11 days. 10% and 40% with ++, +, or − have a similar significance except that the dose would be, respectively, 20,000 and 5,000 parasites in chick cells, and 1,000,000 and 250,000 parasites in duck cells. ++, +, or − alone have the same significance as in Table III. In 1 *a*, *b*, *c* on the 4th day, inoculations were made with undiluted material as well as with a dilution testing for 10 per cent survival.

† The number of exflagellants counted in a 5 minute examination of a fresh preparation is given, except that, if none were found on the 5th and 6th days, the time of examination was extended to 10 minutes.

consistently higher parasite counts during the first 3 days. Preparation 1 *e* is of special interest. The parasite suspension was here included in a clot consisting of equal parts red cell-embryo extract and heparinized plasma. The

TABLE VII

Effects of the Concentration of Plasma, and of Chick Embryo and Yeast Extracts, on Survival of Plasmodium lophurae

No.	Medium	Parasites per c. mm.	Red cells per c. mm.	Infectivity* after days:				Exflagellants† after days:						Parasites per 1000 red cells after days:			
				3	4	5	6	1	2	3	4	5	6	0	1	2	3
2 *a*	Red cell extract—6 ml. frozen-thawed cells in 8 ml. citrated plasma (1 part citrate solution K to 3 parts blood).	.43 × 10^5	2 × 10^5	20% ++	10% +	+	−	1	2	2	1	0		220	232	182	116
2 *b*	Red cell extract using same cells and plasma as for 2 *a*—6 ml. frozen-thawed cells in 5.4 ml. citrated plasma + 2.6 ml. citrate solution K.	.43 × 10^5	2 × 10^5	20% ++	10% ++	+	++	3	1	4	4	0		220	222	192	98
2 *c*	Red cell extract—9 ml. frozen-thawed cells in 5 ml. citrated plasma + 10 ml. citrate solution K.	.43 × 10^5	2 × 10^5	20% ++	10% +	+	+	2	1	0	0			220	216	170	86
2 *d*	Same as 2 *c*, but with 10% by volume of chick embryo extract in citrate solution K.	.43 × 10^5	2 × 10^5	20% ++	10% ++	++	+	1	5	1	0			220	218	180	126
2 *e*	Red cell extract—11 ml. frozen-thawed cells in 5 ml. citrated plasma + 10 ml. citrate solution K. To 5 ml. extract added 0.6 ml. yeast extract.	.43 × 10^5	2 × 10^5	20% ++	10% +	+	−	0	3	1	0			220	234	156	116
2 *f*	Same as 2 *e*, but to 5 ml. red cell extract added 0.1 ml. yeast extract.	.43 × 10^5	2 × 10^5	20% ++	10% ++	++	+	3	0	1	0			220	236	168	126
2 *g*	Red cell extract similar to that for 2 *e* but using blood from other chicks. To 5.5 ml. extract added 0.01 ml. yeast extract.	.43 × 10^5	2 × 10^5	20% ++	10% +	++	+	2	3	0	0			220	214	174	88
3 *a*	Duck red cell extract—4 ml. frozen-thawed cells in 7 ml. citrated plasma (1 part citrate solution K to 3 parts duck blood). Parasites from duck.	.6 × 10^5	1.2 × 10^5	20% +	++	+	−	3	16	8	14	2	0	570	524	346	
3 *b*	Duck red cell extract using same cells and plasma as for 3 *a*—4 ml. frozen-thawed cells in 4.7 ml. citrated plasma + 2.3 ml. citrate solution K.	.6 × 10^5	1.2 × 10^5	20% ++	+	+	−	15	30	8	7	6	0	570	500	340	
3 *c*	Duck red cell extract—12 ml. frozen-thawed cells in 5 ml. citrated plasma + 10 ml. citrate solution K.	.6 × 10^5	1.2 × 10^5	20% ++	−	−		15	5	1	0			570	474	306	
3 *d*	Same as 3 *c*, but with 10% by volume of chick embryo extract in citrate solution K.	.6 × 10^5	1.2 × 10^5	20% +	+	−		6	7	7	0	0		570	516	296	

*, † See corresponding footnotes for Table VI.

clot was covered with liquid red cell-embryo extract which was renewed daily. During the first 4 days small samples were removed from the clot and used to make smears. On the 5th day the clot was ground up and tested for infec-

tivity. This proved to be ++. A large number of exflagellants was observed on this day. This preparation gave high relative parasite counts, the count on the 2nd day being essentially the same as in the original parasite suspension. Similar preparations have consistently given similarly good results, but entirely liquid preparations are much easier to handle and are preferable for comparative tests of various ingredients of the medium.

TABLE VIII

Effects of Different Concentrations of Liver Extract on Survival of Plasmodium lophurae

No.	Medium	Parasites per c. mm.	Red cells per c. mm.	Infectivity* after days:				Exflagellants† after days:					Parasites per 1000 red cells after days:		
				3	4	5	6	1	2	3	4	5	0	1	2
4 *a*	Red cell extract—17 ml. frozen-thawed cells in 27 ml. citrated plasma + 13.5 ml. citrate solution K. To 5.7 ml. extract added 0.3 ml. citrate solution K.	.35 × 10^5	1.9 × 10^5	20% +	10% −	−		3	4	6	2	0	194	176	92
4 *b*	5.7 ml. same red cell extract + 0.29 ml. citrate solution K + 0.01 ml. liver extract in citrate solution K.	.35 × 10^5	1.9 × 10^5	20% +		−		9	18	12	2	0	194	162	134
4 *c*	5.7 ml. same red cell extract + 0.27 ml. citrate solution K + 0.03 ml. liver extract.	.35 × 10^5	1.9 × 10^5	20% +	10% +	−		6	12	2	2	0	194	142	116
4 *d*	5.7 ml. same red cell extract + 0.2 ml. citrate solution K + 0.1 ml. liver extract.	.35 × 10^5	1.9 × 10^5	20% ++	10% −	−		3	1	0	0		194	124	140
4 *e*	5.7 ml. same red cell extract + 0.3 ml. liver extract.	.35 × 10^5	1.9 × 10^5	20% +	10% −	−		0	0	0	0		194		
4 *f*	5.1 ml. same red cell extract + 0.6 ml. embryo extract + 0.29 ml. citrate solution K + 0.01 ml. liver extract.	.35 × 10^5	1.9 × 10^5	20% +	10% −	+	−	1	32	8	0	0	194	176	106
5 *a*	Duck red cell extract—23 ml. frozen-thawed cells in 30 ml. citrated duck plasma + 15 ml. citrate solution K. To 5.7 ml. extract added 0.3 ml. citrate solution K. Parasites from duck.	.87 × 10^5	1.3 × 10^5	++	++	+		2	6	2	0		720	608	404
5 *b*	5.1 ml. same red cell extract + 0.6 ml. chick embryo extract + 0.3 ml. citrate solution K.	.87 × 10^5	1.3 × 10^5	++	+	+		8	5	4	2	0	720	636	468
5 *c*	Same as 5 *b* but 0.29 ml. citrate solution K and 0.01 ml. liver extract.	.87 × 10^5	1.3 × 10^5	++	+	+		1	15	8	0	0	720	682	448
5 *d*	Same as 5 *b* but 0.25 ml. citrate solution K and 0.05 ml. liver extract.	.87 × 10^5	1.3 × 10^5	++	−			7	3	1			720		
5 *e*	Same as 5 *b* but 0.3 ml. liver extract.	.87 × 10^5	1.3 × 10^5	−	−			5	0	0			720		

*, † See corresponding footnotes for Table VI.

Table VII, 2 *a–c*, shows that red cell extract prepared in about equal parts of plasma and citrate solution K (*b*) was better than a similar extract in whole citrated plasma (*a* — one part citrate solution K to 3 parts plasma) or an extract in one part of plasma to 3 parts of citrate solution K (*c*). The results on infectivity, exflagellation, and parasite number are all in fair agreement. However, although the infectivity of preparation 2 *c* was slightly better than

that of 2 *a*, the reverse was true of the exflagellation. Preparations 3 *a–c* were similar to 2 *a–c* but with duck material. Here the highest plasma concentration proved to be as good as, or perhaps slightly better than, the intermediate concentration, while the lowest concentration was definitely worse in all respects.

Preparations 2 *c*, *d* illustrate the consistently favorable effect of the addition of chick embryo extract to the medium at a concentration not exceeding 10 per cent by volume. Higher concentrations were deleterious. The effect is also shown by preparations 3 *c*, *d*, and by 4 *b*, *f* of Table VIII. Preparations

TABLE IX

The Survival of Plasmodium lophurae in Concentrated Red Cell Extracts with and without Liver Extract

No.	Medium	Parasites per c. mm.	Red cells per c. mm.	Infectivity* after days:				Exflagellants† after days:					Parasites per 1000 red cells after days:		
				3	4	5	6	1	2	3	4	5	0	1	2
6 *a*	Red cell extract—14 ml. frozen-thawed cells in 7 ml. serum + 7 ml. solution K. To 6 ml. extract containing some of the nuclear residue added 0.6 ml. embryo extract.	.3 × 10^5	2.4 × 10^5	40% +	20% +	++	+	4	10	9	17	0	134		
6 *b*‡	Red cell extract—12 ml. frozen-thawed cells in 6 ml. serum + 6 ml. solution K. To 6 ml. extract containing a light suspension of free nuclei added 0.6 ml. embryo extract + 0.01 ml. liver extract.	.3 × 10^5	2.4 × 10^5	40% ++	20% ++	+		6	2	5	0	0	134	178	148
7 *a*	Duck red cell extract—14 ml. frozen-thawed cells in 7.5 ml. duck serum + 7.5 ml. solution K. To 6 ml. extract containing some of nuclear residue added 0.6 ml. chick embryo extract. Parasites from duck.	.8 × 10^5	1.5 × 10^5	20% ++	10% ++	++	−	18	13	12	3	1	568		
7 *b*	Duck red cell extract—12 ml. frozen-thawed cells in 6 ml. duck serum + 6 ml. solution K. To 7 ml. clear extract added 0.7 ml. chick embryo extract + 0.01 ml. liver extract.	.8 × 10^5	1.5 × 10^5	20% ++	10% +	+	−	26	12	17	5	15	0	568	604	532

*, † See corresponding footnotes for Table VI.

‡ This preparation was accidentally contaminated on the 5th day.

2 *e*, *f*, *g* show the effects of a yeast extract prepared by boiling 2.5 gm. of dried Harris brewers' yeast in 25 ml. of citrate solution K, filtering through paper, and sterilizing by Berkefeld filtration. Of the three concentrations tried, the intermediate one was slightly better than the others.

The effects of the addition of chicken liver extract using chick and duck material are shown by series 4 *a–e* and 5 *b–e* respectively of Table VIII. In

these experiments equal parts of plasma and citrate solution K were used in the preparation of dilute red cell extracts, and the survival was not as good as in the preceding series, employing more concentrated red cell extracts. In the chick series, the lowest concentration of liver extract tried (4 *b*) definitely enhanced the exflagellation. The chicks used for the 4th day infectivity test on this preparation both died before results could be obtained from them, but the preparation (4 *c*) with the next higher concentration of liver extract showed a better infectivity than the preparation without liver extract. Still higher concentrations of liver extract were toxic. With the duck material the lowest concentration of liver extract did slightly enhance the exflagellation on the 2nd and 3rd days, but no effect on infectivity was observed. The higher concentrations were again toxic.

Preparations 6 *a*, *b*, and 7 *a*, *b* of Table IX show the results of tests with as concentrated red cell extracts, made in equal parts of serum and solution K, as could conveniently be prepared in the necessary amount. Preparations 6 *a* and 7 *a* contained in the extract a considerable amount of the nuclear residue, and the presence of these numerous free nuclei precluded accurate parasite counts. Preparation 6 *b* contained some free nuclei and preparation 7 *b* very few. In preparation 6 *b*, 40 per cent or more of the original parasites were still alive on the 3rd day and 20 per cent or more on the 4th day. The parasite number increased by a significant amount after 1 day's incubation, but by the 2nd day it had dropped back to about the original figure. No significant decrease in total red cell count was observed in this preparation until the 4th day. In preparation 7 *b* the parasite number maintained itself for the first 2 days, with a possible increase on the 1st day. In this preparation, the red cell count went down after the 2nd day. Duck red cells have typically proved to be less resistant to the *in vitro* conditions than have chick red cells. It is worth noting that in preparation 7 *b* as many as 15 exflagellants were found in 5 minutes in a sample taken after 5 days of life *in vitro* at a temperature of almost 42°C.

DISCUSSION

Only two questions need be discussed here. Were the various effects which were observed direct effects of the environmental conditions on the malaria parasites, or were they indirect effects *via* the red cells in which the parasites were contained? Did the parasites which survived in these experiments continue to develop while surviving? To the first question no answer can be given. Since there is as yet no way of maintaining the parasites outside of red cells, the effects of environmental conditions on the two cannot be readily separated. It can be said that many normal appearing erythrocytes were still present in preparations in which the parasites had died out. Also, dextrose appears to have no effect on the respiration of normal chicken erythrocytes (29), suggesting that its effect on survival of *P. lophurae* was a direct one.

With respect to the second question, the evidence indicates that the parasites surviving *in vitro* continue to develop, especially during the first few days. In the most favorable survival tests the parasite number maintained itself during the first 2 days and, rarely, increased slightly during the 1st day. Moreover, the percentage of very young forms devoid of pigment, and of segmenters, varied from day to day. The finding of appreciable numbers of very young forms, as well as segmenters and intermediate forms (Fig. 2), after 3 or 4 days *in vitro*, is considered to be an indication that the infection of new red cells occurred. The problem encountered by Hewitt (11) of the failure of merozoites to break out of the host cell did not arise in the present studies with *P. lophurae*. Numerous free merozoite clusters could be found. The presence of fairly numerous very young forms, as well as segmenters, was generally correlated with good survival as judged by infectivity. On the other hand, the presence of many segmenters with very few young forms was usually accompanied by relatively poor survival as judged by infectivity. It is unfortunate that *P. lophurae* exhibits such a low synchronicity (24) that more accurate data on these points are difficult to obtain. The repeated observation of more exflagellants on the 2nd, 3rd, 4th, or even 5th day of life *in vitro* than on preceding days again suggests that development of the male gametocytes occurred *in vitro* and that we are not dealing with a mere survival of gametocytes already ripe at the time of preparation of the survival tests. Indeed, the conditions of preparation were usually such that the parasite suspension was exposed to room temperature for more than 20 minutes and active exflagellation of the male gametocytes, already ripe at the time of preparation, occurred in it.

SUMMARY

The survival of *Plasmodium lophurae in vitro*, at temperatures of 39.5–42°C., is favored by a balanced salt solution having a high potassium content, by aeration but not by a very high oxygen tension, by an optimal density of parasites per cubic millimeter, by frequent renewal of the suspending medium, by concentrated red cell extract, by optimal concentrations of plasma or serum, of chick embryo extract, of glucose or glycogen, and of glutathione, and probably by yeast extract and a very low concentration of liver extract.

In the best preparations, as judged by infectivity, more than 40 per cent of the parasites were alive on the 3rd day, more than 20 per cent on the 4th day, perhaps 1 per cent on the 5th day, and about 0.05 per cent on the 6th day. Evidence was obtained that the parasites had multiplied during the 1st day of incubation.

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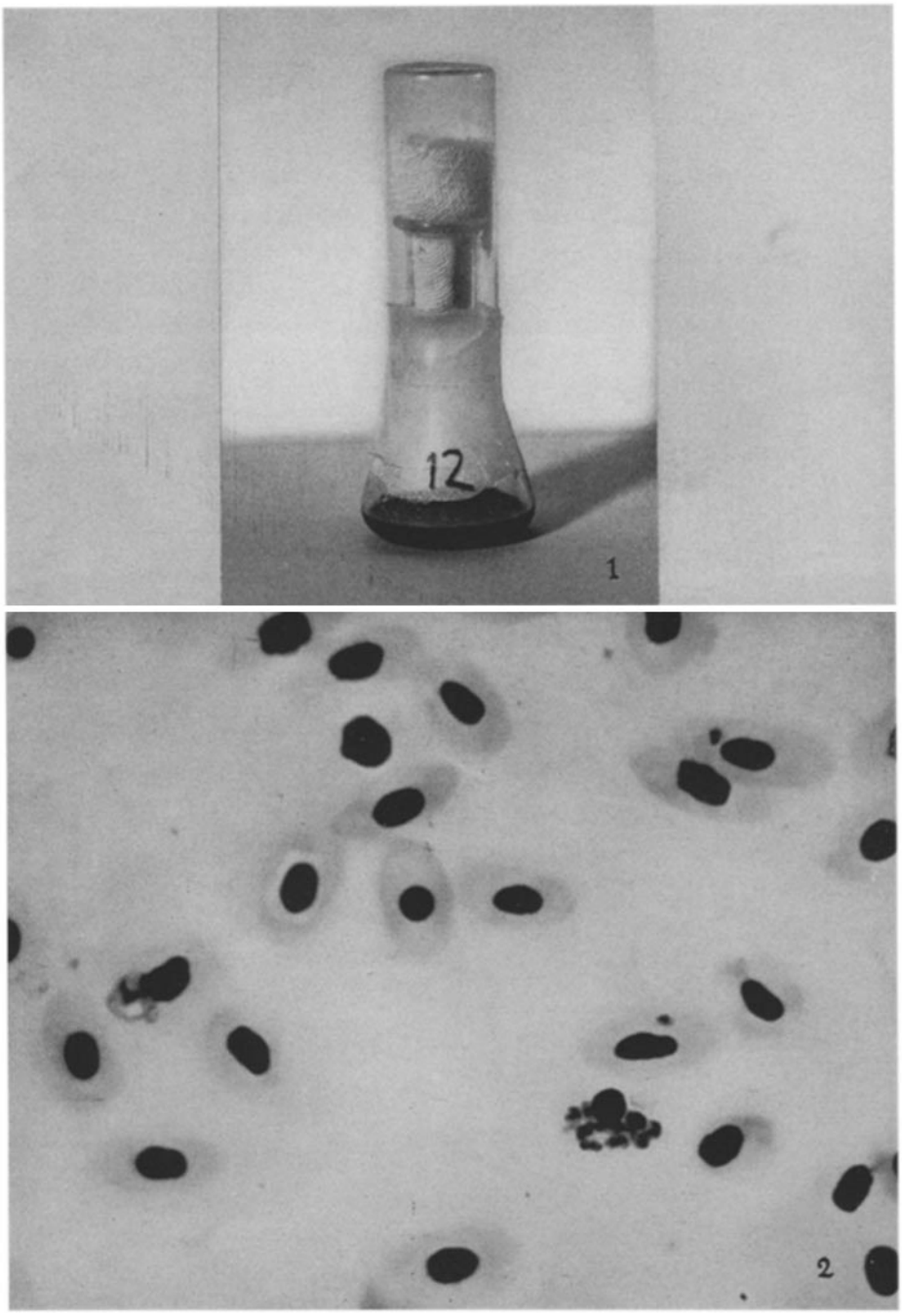
EXPLANATION OF PLATE 23

FIG. 1. 25 ml. Erlenmeyer flask holding 2 ml. of medium and capped with vial sealed with parafilm, as used for most of the survival tests. × 0.6.

FIG. 2. Portion of a Giemsa-stained smear from a survival test after 3 days of incubation at 41.5–42°C. (Preparation 2*b* of Table VII.) Note the segmenter, two young forms, and the delicately stained large trophozoite. × 1569.

Photographed by Julian A. Carlile.



(Trager: Conditions affecting survival of malarial parasite)